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Two E2 Binding Sites Alone Are Sufficient To Function as the Minimal Origin of Replication of Human Papillomavirus Type 18 DNA

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Replication of papillomaviruses requires an origin of replication and two virus-encoded proteins, E1 and E2. Using a transient replication assay for human papillomavirus type 18 (HPV-18) DNA, we have found that two adjacent sequences present within the origin of replication can independently support replication. The first, a 77-bp region, contains one E2 binding site (E2BS) and a 16-bp inverted repeat element that probably corresponds to the E1 binding site (E1BS). The other, an 81-bp region, includes two E2BS but lacks the putative E1BS. A synthetic 33-bp oligonucleotide containing two high-affinity E2BS was also found to function as an origin of replication. Replication of all these plasmids was absolutely dependent on the presence of the HPV-18 E1 and E2 proteins. The HPV-1a E1 and E2 proteins were also found to support replication of a plasmid containing the complete HPV-18 origin but failed to replicate a plasmid containing two E2BS alone. Our results suggest that the E2 protein can target E1 to the origin through the formation of an E1-E2 complex which is likely to be involved the initiation of replication.

Papillomaviruses are small DNA viruses which infect humans and a wide range of animals. These viruses induce benign proliferative squamous epithelial and fibroepithelial lesions (warts and papillomas) in their natural hosts. Human papillomaviruses (HPVs) such as type 16 (HPV-16) and HPV-18 are involved in the pathogenesis of anogenital cancer (7, 21, 38). In benign tumors, the viral DNA is present exclusively in an episomal (extrachromosomal) form, but most malignant tumors contain integrated viral DNA (27, 38). Because of their stable, extrachromosomal replication, papillomaviruses provide useful model systems with which to study DNA replication in eukaryotic cells (16). Transient replication analysis and in vitro studies of bovine papillomavirus type 1 (BPV-1) and various HPV DNAs has revealed the requirement of two viral proteins, E1 and E2, in replication (3, 4, 6, 16, 25, 30–36). We have recently shown that in the case of HPV-1a, E1 alone is sufficient for transient replication (9). The origin of replication (ori) of papillomaviruses is present within the viral long control region (LCR) and contains one E1 binding site (E1BS) and one or more E2 binding sites (E2BS). The E1 protein has ATPase, DNA helicase, and DNA binding activities (14, 18, 29, 30, 32, 36, 37). The BPV-1 E1 protein binds to a palindromic 18-bp inverted repeat (IR) element centered around the *Hpa*I or *Hpa*I-like sequence (14, 18, 33, 36). The E2 protein is an activator as well as a repressor of viral transcription. The E2 protein binds to palindromic $ACCGN_4CGGT$ sequences present within the LCR of the viral DNA (1, 8, 11, 12, 17, 20, 23). This sequence motif is thought to represent high-affinity E2BS, while the sequence $ACCN_6GGT$ corresponds to weak E2BS (1, 17). The E2 protein of BPV-1 forms a specific complex with E1 and enhances its binding to the origin (19, 22, 28, 30). Previous studies on the transient replication of HPV-18 DNA have identified a 177-bp region containing one putative E1BS and three E2BS that are sufficient for maximal replication (25, 31). We have further analyzed the roles of various sequence elements present within the HPV-18 origin in DNA replication.

Delineation of the HPV-18 ori. To determine the roles of various *cis* elements in HPV-18 replication, various plasmids containing different portions of the origin were generated. Plasmids pori177, pori117, pori108, and pori63 have been previously described (31) and were included in the current studies for comparison purposes. Various deletion plasmids were generated by cloning restriction fragments internal to the HPV-18 LCR into plasmid pUC19 (26). The deletion endpoints of the cloned DNAs are shown in Fig. 1A. The ends of the restriction fragments were filled in with the Klenow fragment of DNA polymerase I where necessary (26). Plasmid pori127 contains a 127-bp *Bbv*I-*Bam*HI fragment (nucleotides [nt] 7850 to 7857 and 1 to 119 of HPV-18) cloned into the *Hin*cII-*Bam*HI sites of pUC19. Plasmid pori104 contains a 104-bp *Mun*I-*Bam*HI fragment (nt 16 to 119) cloned into the *Eco*RI-*Bam*HI sites of pUC19. Plasmid pori81 contains a 81-bp *Mae*III-*Bam*HI fragment (nt 39 to 119) cloned into the *Hin*cII-*Bam*HI sites of pUC19. Plasmid pori117* contains a 117-bp *Alu*I-*Ava*II fragment (nt 7800 to 7857 and 1 to 59) cloned into the *Hin*cII site of pUC19. Plasmid pori101 contains a 101-bp *Alu*I-*Mae*III fragment (nt 7800 to 7857 and 1 to 43) ligated into the *Hin*cII site of pUC19. Plasmid pori77 contains a 77-bp *Alu*I-*Mun*I fragment (nt 7800 to 7857 and 1 to 19) ligated into the *Hin*cII-*Eco*RI sites of pUC19. Plasmid pori62 was generated by ligating a 62-bp *Hin*dIII-*Ase*I fragment from pori177 containing nt 7800 to 7857 and 1 to 4 of HPV-18 into the *Hin*cII site of pUC19. Plasmids pori57, pori48, and pori41 were generated by inserting a 57-bp *Ase*I-*Ava*II fragment (nt 3 to 59), a 48-bp *Mse*I-*Ava*II fragment (nt 12 to 59), and a 41-bp *Ase*I-*Mae*III

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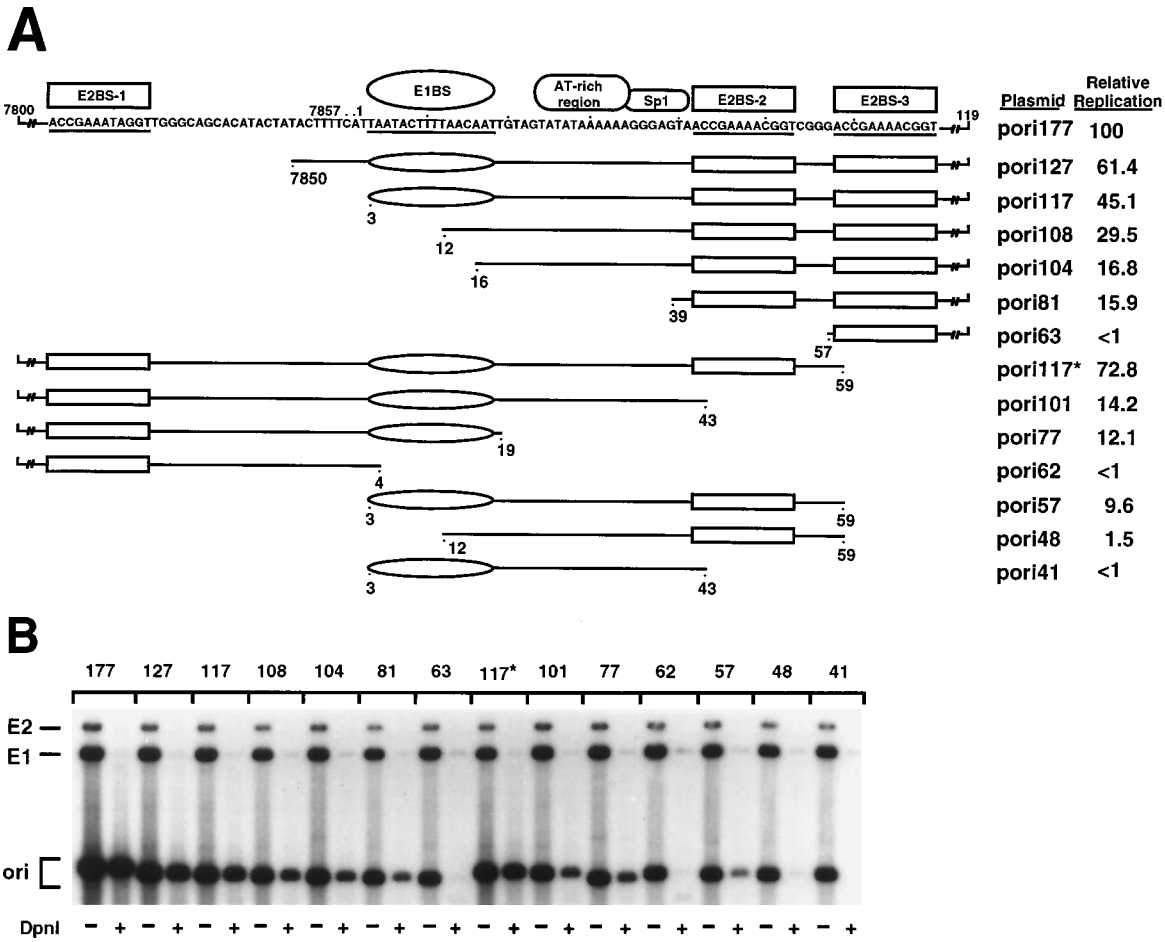

FIG. 1. Functional mapping of the HPV-18 ori. (A) The boundaries of the various ori plasmids used in transient replication assays are shown. The location of the imperfect 16-bp IR element containing the *Hpa*I-like sequence that probably corresponds to the E1BS is shown. Also shown are the E2BS, the A/T-rich region, and the putative Sp1 binding site. The map coordinates correspond to the published sequence of HPV-18 (5). The relative replication efficiency of each plasmid with respect to that obtained with pori177 is indicated and was determined by measuring the radioactivity in filter strips, using a scintillation counter. (B) Transient replication analysis of the various plasmids in C-33A cells. Low-molecular-weight DNA was isolated and analyzed by Southern blot hybridization as described in the text. The Hirt fractions were treated with *Eco*RI, and one half of each sample was then digested with *Dpn*I to remove the unreplicated DNA. The relative levels of replication in each sample were measured by normalizing the radioactivity in the origin fragments in lanes treated with *Dpn*I to that present in the E1 band in lanes untreated with *Dpn*I. This was done to account for minor differences in the recovery of various samples. Plasmids pSGE1 and pSGE2 in lanes treated with *Dpn*I also serve as internal controls since they lack the origin and do not replicate. The positions of the various ori plasmids and the E1- and E2-expressing plasmids are indicated. The autoradiogram was exposed for 1 h.

fragment (nt 3 to 43), respectively, into the *Hin*cII site of pUC19.

Transient replication analysis was carried out with the human C-33A cervical carcinoma cell line. These cells have previously been shown to efficiently support transient replication of HPVs (3, 4, 31). Plasmid transfections were carried out by calcium phosphate coprecipitation (2), and the efficiency of transfection was approximately 25%. One-half microgram of plasmid pori177 or an equimolar amount of each of the other ori plasmids was transfected into C-33A cells along with 5 μg of pSGE1 and 1 μg of pSGE2, expressing the HPV-18 E1 and E2 proteins, respectively (10, 31). These conditions have previously been shown to be optimal for HPV-18 replication (31). Three days posttransfection, Hirt fractions containing the low-molecular-weight DNA were isolated (13). The samples were treated with *Eco*RI to linearize the various plasmid DNAs except for pSGE1, which was cleaved into two fragments, only one of which is complementary to the probe. Plasmids pori77 and pori104 were linearized with *Hin*dIII since they lack an *Eco*RI site. One half of each sample was treated with *Dpn*I to distinguish between input and replicated DNA (24). The DNA samples were analyzed by agarose gel electrophoresis and Southern blot hybridization (26, 31). ^{32}P-labeled pUC19 DNA was used as the probe, and the blots were subjected to autoradiography. The autoradiogram shown in Fig. 1B is representative of three independent experiments, all of which gave similar results. The extent of replication was determined by measuring the radioactivity in filter strips by liquid scintillation counting. No replication was observed in the absence of plasmid pSGE1 or pSGE2, and the vector plasmid pUC19 did not replicate (data not shown). As shown earlier, plasmid pori177, containing the imperfect 16-bp IR element and three E2BS, showed maximal replication (Fig. 1). The level of replication of pori177 was similar to that obtained with plasmid pUCLCR-18, containing the complete LCR of HPV-18 (not shown). Interestingly, plasmids pori81 and pori104 replicated to a significant extent (~16% of the maximal levels) (Fig. 1). These plasmids contain two high-affinity E2BS but lack the 16-bp IR element surrounding the *Hpa*I-like sequence. DNA binding experiments with the BPV-1 E1 protein suggest that its binding

site is centered around an 18-bp IR element, termed the *Hpa*I or *Hpa*I-like region, present within the BPV-1 ori (14, 30). A consensus sequence, T-RY--TTAA--RY-A, has been identified in similar regions of many other papillomaviruses (14). The E1 protein of HPV-11 is also known to bind to DNA fragments that contain this IR region (18). The 16-bp IR element of HPV-18 (nt 3 to 18) was found to significantly stimulate replication, since pori117 replicated to ~3-fold higher levels than pori104 (Fig. 1B). This region includes the *Hpa*I-like sequence and is postulated to be the binding site of the E1 protein of papillomaviruses (14). Although we have not studied the binding of the HPV-18 E1 protein to the DNA, it is likely that the 16-bp IR region corresponds to the E1BS. Also, since pori127 replicated to ~33% higher levels than pori117 (Fig. 1), it is possible that the putative E1BS extends beyond this IR region. The replication of plasmid pori81 in the presence of E1 and E2 proteins suggests that a DNA containing two E2BS can constitute a functional HPV-18 ori (see below). Plasmids pori63 and pori62, containing only one E2BS, did not replicate. Plasmids pori101, pori77, and pori57, containing the 16-bp IR element and one E2BS, also replicated to significant levels (Fig. 1). However, plasmid pori41, containing the putative E1BS but lacking the E2BS, did not replicate. These results suggest that DNA containing the 16-bp IR element alone does not constitute a functional origin, but the presence of one E2BS along with the putative E1BS can support HPV-18 DNA replication. Another important conclusion can be drawn from these results. Plasmids pori77 (nt 7800 to 7857 and 1 to 19) and pori81 (nt 39 to 119) replicated to approximately similar levels. Since these plasmids contain nonoverlapping portions of the HPV-18 origin (Fig. 1A), the results presented above suggest that replication can initiate from two separate regions of the origin. Also, since an E2BS is common to both pori77 and pori81, it is possible that the E2BS is involved in the initiation of replication. The HPV-18 origin contains an A/T-rich region and a putative Sp1 binding site immediately adjacent to the 16-bp IR element (Fig. 1A). Deletion of these sequences did not affect replication (compare pori104 with pori81 and pori101 with pori77). These results suggest that the A/T-rich region and the Sp1 binding site do not play an important role in replication in the context of the ori plasmids used in our studies.

We tested the effect of the location of the E2BS relative to the 16-bp IR element on transient replication. The HPV-18 origin contains three E2BS (Fig. 1A). E2BS-2 and E2BS-3 represent high-affinity sites having the sequence motif AC CGN_4CGGT, whereas E2BS-1 corresponds to a lower-affinity site with the motif $ACCN_6GGT$ (1, 17). Plasmids pori57 and pori77 contain one E2BS which is present on either side of the putative E1BS (Fig. 1A). These plasmids replicated to similar levels (within approximately 1.3-fold of each other) even though pori77 contains a presumably weaker E2BS having the $ACCN_6GGT$ motif. These results indicate that the relative location of the E2BS with respect to the putative E1BS and minor differences in the consensus sequence of the E2BS do not appear to significantly affect HPV-18 replication. This observation is in contrast to the recent results obtained with the HPV-11 system; in this case, a 10- to 20-fold difference in replication was seen when plasmids containing the E1BS contained a high-affinity E2BS on either side (18). Differences in adjacent sequences, the respective E1 and E2 proteins, or the local DNA structure could account for the results obtained with these two systems. Also, with plasmids containing two E2BS in addition to the 16-bp IR element (pori127 and pori117*), the relative location of the E2BS with respect to the IR element did not appreciably affect replication (Fig. 1).

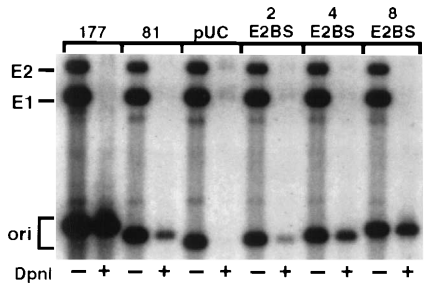

FIG. 2. Replication of plasmids containing synthetic E2BS by HPV-18 E1 and E2 proteins. One-half microgram of pUC19, pori177, pori81, or plasmids containing various number of synthetic E2BS was transfected into C-33A cells along with 5 μg of pSGE1 and 1 μg of pSGE2. The positions of the various plasmid bands are indicated as for Fig. 1B.

Two E2BS alone are sufficient to support HPV-18 replication. The foregoing results showed that the pori81 DNA containing two E2BS was sufficient for replication. However, since the binding site of the HPV-18 E1 protein has not been experimentally determined, we generated plasmid pUC7 derivatives containing synthetic E2BS by inserting into the *Hin*cII site of pUC7 a 33-bp oligonucleotide having the following sequence:

5′ GGACCGAAAACGGTTCAACCGAAAACGGTTGTA 3′
3′ CCTGGCTTTTGCCAAGTTGGCTTTTGCCAACAT 5′

This sequence contains two high-affinity E2BS (underlined) and is unlikely to contain a binding site for the HPV-18 E1 protein. Plasmids containing one, two, and four inserts (corresponding to two, four, and eight E2BS) were obtained and used in replication studies. A plasmid containing two E2BS replicated to detectable levels (Fig. 2). Furthermore, plasmids containing four or eight adjacent E2BS replicated to much higher levels than those containing two E2BS (Fig. 2). These results suggest that two E2BS alone are capable of supporting specific replication in the presence of the HPV-18 E1 and E2 proteins. Furthermore, the presence of additional E2BS results in a further increase in the efficiency of replication. Recently, it has been shown with the HPV-11 system that one E1BS along with one E2BS, or two E2BS alone, can support transient replication (18). Studies with BPV-1 have also shown that mutant origins that have greatly reduced ability to bind E1 can support replication in the presence of the E2 protein (30). These studies suggested that efficient replication in the absence of E1 binding to the origin can occur as a result of the binding of the E1-E2 complex to the origin. Our results are consistent with this hypothesis, and it is likely that initiation of replication from plasmids containing two E2BS alone results from the stabilization of E1 to the DNA by the E2 protein.

Replication of HPV-18 ori plasmids by HPV-1a E1 and E2 proteins. We have recently described a system for the replication of HPV-1a ori DNA in transiently transfected C-33A cells (9). Previous studies with various papillomaviruses have shown a lack of specificity of the viral E1 and E2 proteins in the replication of heterologous ori DNAs (4, 6, 9, 31). We tested the ability of the HPV-1a E1 and E2 proteins to replicate various HPV-18 ori DNAs. These proteins supported replication of the pori177 plasmid to levels similar to those obtained with the HPV-18 E1 and E2 proteins (Fig. 3). These results showed that the HPV-1a E1 and E2 proteins interact efficiently with the heterologous HPV-18 origin. On the other hand, the ability of HPV-1a E1 and E2 proteins to replicate several HPV-18 ori plasmids lacking one or more E2BS was greatly

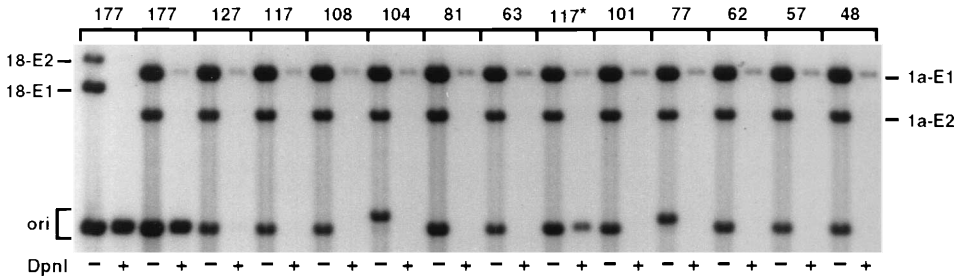

FIG. 3. Replication of HPV-18 ori plasmids by HPV-1a E1 and E2 proteins. C-33A cells were transfected with 0.5 μg of pori177 or equimolar amounts of the various ori plasmids along with 8 μg of pSGE1-1a and 1 μg of pSGE2-1a. In a control experiment, pori177 was cotransfected into C-33A cells with 5 μg of pSGE1-18 and 1 μg of pSGE2-18. The samples were treated as described in the legend to Fig. 1B and in the text. The positions of the HPV-18 and HPV-1a E1- and E2-expressing plasmids are indicated.

reduced or abolished. The only deletion plasmid that replicated to a significant level was pori117*, while pori127 gave a very faint signal upon overexposure of the autoradiogram (Fig. 3). Since both of these plasmids contain two E2BS (pori127 contains two high-affinity E2BS, while pori117* contains one low-affinity and one high-affinity site), these results suggest that replication of HPV-18 ori DNA by the heterologous HPV-1a E1 and E2 proteins is sensitive to the relative location of the E2BS with respect to the 16-bp IR element. Plasmids containing two E2BS (pori108, pori104, and pori81) failed to replicate to detectable levels (Fig. 3). Plasmids containing two, four, or eight synthetic E2BS also did not replicate (data not shown). These results show that two E2BS alone do not constitute a functional ori in the presence of the E1 and E2 proteins from papillomaviruses such as HPV-1a. These results along with previous studies (9, 18) suggest that there may be interesting differences between the sequence requirements for origin function in the case of HPVs that cause mucosal lesions (such as HPV-11 and HPV-18) and those that cause cutaneous lesions (such as HPV-1a). This postulate is supported by the observations that while two E2BS alone can function as oris in the presence of the E1 and E2 proteins of HPV-11 and HPV-18, this is not the case with the HPV-1a E1 and E2 proteins. The E1 and E2 proteins of HPV-1a also did not support replication of HPV-18 ori plasmids containing the 16-bp IR element along with either one E2BS (pori101, pori77, and pori57) or with two E2BS located on the same side of the IR element (pori117) (Fig. 3). Thus, although the HPV-1a E1 and E2 proteins can efficiently replicate plasmids containing the complete HPV-18 origin, the requirement and relative locations of the E1 and E2 binding sites for replication are more stringent than those for the homologous E1 and E2 proteins. In most samples, weak *Dpn*I-resistant signals were observed for plasmid pSGE1-1a (Fig. 3). However, this was not reproducible and may have been due to the higher levels of this plasmid used compared with the ori plasmids.

In summary, results presented in this study suggest that two adjacent sequences present within the HPV-18 origin (nt 7800 to 7857 and 1 to 19 and nt 39 to 119) can independently function as oris. One origin includes the putative E1BS and one E2BS, while the other contains two E2BS. This conclusion is strengthened by the observation that the HPV-18 E1 and E2 proteins can support replication of plasmids containing two synthetic E2BS alone. In plasmids containing multiple E2BS along with the putative E1BS, the relative location of the E2BS with respect to the E1BS did not significantly affect the efficiency of replication in the presence of HPV-18 E1 and E2 proteins. Although the HPV-1a E1 and E2 proteins support efficient replication of plasmid pori177, containing the complete HPV-18 origin, they do not replicate plasmids containing two E2BS alone or the 16-bp IR element plus one E2BS. These results suggest that the ability of the HPV-1a E1 and E2 proteins to support replication from the heterologous HPV-18 origin is much more sensitive to variations in the number and location of the E2BS within the origin. Also, our results reveal that not all of the papillomavirus E1 and E2 proteins are capable of supporting replication of plasmids containing multiple copies of the highly conserved E2BS.

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